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The role of cell seeding density and nutrient supply for articular cartilage tissue engineering with deformational loading

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Summary

Objective: Functional tissue engineering (FTE) of articular cartilage involves the use of physiologically relevant mechanical signals to encourage the growth of engineered constructs. The goal of this study was to determine the utility of deformational loading in enhancing the mechanical properties of chondrocyte-seeded agarose hydrogels, and to investigate the role of initial cell seeding density and nutrient supply in this process.

Design: Chondrocyte-seeded agarose hydrogels were cultured in free-swelling conditions or with intermittent deformational loading (10% deformation, 1 Hz, 1 h on/1 h off, 3 h per day, five days per week) over a two-month culture period. Disks were seeded at lower (10 million cells/ml) and higher (60 million cells/ml) seeding densities in the context of a greater medium supply than previous studies (decreasing the number of cells/ml feed medium/day) and with an increasing concentration of fetal bovine serum (10 or 20% FBS).

Results: Under these more optimal nutrient conditions, at higher seeding densities and high serum concentration (20% FBS), dynamically loaded constructs show >2-fold increases in material properties relative to free-swelling controls. After two months of culture, dynamically loaded constructs achieved a Young's modulus of ~185 kPa and a dynamic modulus (at 1 Hz) of ~1.6 MPa, with a frequency dependent response similar to that of the native tissue. These values represent ~3/4 and ~1/4 the values measured for the native tissue, respectively. While significant differences were found in mechanical properties, staining and bulk measurements of both proteoglycan and collagen content of higher seeding density constructs revealed no significant differences between free-swelling and loading groups. This finding indicates that deformational loading may act to increase material properties via differences in the structural organization, the production of small linker ECM molecules, or by modulating the size of macromolecular proteoglycan aggregates.

Conclusions: Taken together, these results point to the utility of dynamic deformational loading in the mechanical preconditioning of engineered articular cartilage constructs and the necessity for increasing feed media volume and serum supplementation with increasing cell seeding densities.

Introduction

Articular cartilage lines the bony surfaces of diarthrodial joints and functions to transmit the high stresses associated with joint loading. The ability of this tissue to function in the demanding environment of the joint is dependent on its unique material properties and dense extracellular matrix (made up mostly of proteoglycans and type II collagen)[1]. The measured compressive mechanical properties of the native tissue depend on age, harvest site, and testing configuration (i.e., confined vs unconfined compression)[1]. For example, for fetal (third trimester) and adult (1–2 years) bovine knee articular cartilage the aggregate modulus has been reported to vary between 0.15–0.38 MPa in confined compression[2,3]. Alternatively, the unconfined compressive Young's modulus of juvenile (2–3 month) cartilage from either the shoulder or wrist has been found to range from 0.29–0.35 MPa[4,5]. There is, likewise, a dependence of the measured biochemical constituents on age, joint and location, with reported values of GAG content ranging from ~2.5–10% of the tissue wet weight[3,6] and collagen content ranging from ~5–30% of the tissue wet weight.[1,3,6] While articular cartilage may function well over a lifetime, traumatic injury or the degenerative changes associated with osteoarthritis (OA) can result in significant erosion of the articular layer, leading to joint pain and instability[7]. The poor healing capacity of the tissue, coupled with continual increases in life expectancy, and the limited number of clinical remedies, create a demand for an engineered articular cartilage source. To this end a number of studies have investigated the growth of articular cartilage *in vitro* and *in vivo*, in a variety of three-dimensional matrices and bioreactor systems[8–22].

Our studies have used the application of physiologic deformational loading to promote the development of articular cartilage constructs having mechanical and biochemical properties similar to that of the native tissue. With this functional tissue engineering (FTE) approach[23,24], we have demonstrated that deformational loading of chondrocyte-seeded agarose hydrogel constructs (10% peak-to-peak deformation, at 1 Hz for three 1-h load/rest cycles) results in significantly enhanced biochemical and material properties relative to unloaded, free-swelling controls[25,26]. As reported in the literature for other cartilage tissue engineering systems[11,15,27–29], tissue elaboration appears to be modulated by cell-seeding density and by medium constituents (e.g., growth factors)[16,26,30]. In our

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previous study, no effect of deformational loading was observed for 60 million cells/ml constructs, whereas a 3-fold enhancement over control was apparent for constructs seeded at 20 million cells/ml. Over the same 28-day culture period, the maximum stiffness achieved for the 60 million cells/ml free-swelling constructs was similar to the peak stiffness measured for the loaded constructs seeded at 20 million cells/ml[26]. We hypothesize that this finding was due to insufficient nutrient availability to the cells in the relatively high seeding density cultures. The earlier positive results were achieved with a ratio of cells/ml feed medium/day of <2 million, compared to a ratio of >3.5 million cells/ml feed medium/day for the 60 million cells/ml constructs. A review of other bioreactors for cartilage tissue engineering (particularly those which seek to optimize nutrient availability) reveals that this ratio is generally maintained at levels less than 1.0-2.2 million cells/ml feed medium/day[8,9,12,13,19,21,22].

In the present study, to further investigate the issue of nutrient supply, we revisit the use of cultures seeded at 60 million cells/ml to determine if conditions can be optimized so that the beneficial effects of deformational loading (observed at lower seeding densities) can be realized at higher seeding densities. Native young bovine articular cartilage has a cell density that ranges from 240–100 million cells/ml through the depth of the tissue (surface to deep) and changes with tissue maturation[31]. For tissue engineering studies, initial seeding densities have ranged from 10–130 million cells/ml[8,11,14,18,22,26]. The hypothesis of this study is that increasing the availability of nutrients (absolute volume of medium and serum concentration) and the number of cells will enhance the development of engineered cartilage constructs in conjunction with deformational loading. This hypothesis was assessed by increasing the medium volume per construct by ~2-fold over our earlier study (to maintain ~1.7 million cells/ml feed medium/day), and by examining the effect of fetal bovine serum (FBS) supplementation of feed medium at either 10 or 20% on construct development (glycosaminoglycan (GAG) and collagen content and distribution, Young's modulus and unconfined dynamic modulus).

Materials and methods

TISSUE ISOLATION AND CELL CULTURE

Cell-seeded agarose hydrogels were prepared as previously described[25,26,32]. Briefly, cartilage was isolated from ten carpometacarpal joints of the forelimbs of a minimum of five young (4–6 month old) calves. The cartilage pieces were combined and digested in DMEM (5 ml per gram of tissue) with 2.5 mg/ml pronase (Calbiochem, San Diego, CA) for 1 h at 37°C with stirring, followed by 0.5 mg/ml collagenase type II (Sigma Chemicals, St. Louis, MO) for 4 h at 37°C with stirring. Cell suspensions were filtered, sedimented at 1000×*g* for 10 min, and then resuspended in fresh DMEM supplemented as described previously[25,26,32]. Cell suspensions were mixed in equal parts with 4% agarose in PBS (Type VII, low gelling temperature) to produce final cell concentrations of either 10 or 60 million cells/ml in 2% agarose. After gelling chondrocyte-seeded slabs at each seeding density at room temperature for 15 min between parallel plates, disks (Ø 4.76×2.2 mm thickness) were cored, and cultured in 100 mm petri dishes (20 to 25 disks per plate) with 30 ml of high glucose DMEM (without serum) with 50 µg/ml fresh ascorbic acid at 37°C and 5% CO_2 for two days to allow cells to adjust to their new culture environment. Afterward, constructs were provided 30 ml medium containing either 10 or 20% FBS from the same lot. This creates eight distinct experimental groups, one for each seeding density, one for each medium concentration, each cultured with (loaded) or without (free-swelling) the application of dynamic deformational loading. Medium was replaced in total each day of loading (see below). Cylindrical full-thickness articular cartilage samples (Ø 4 mm) were also harvested from the same joints for analysis of mechanical and biochemical properties. Subchondral bone was removed in a custom cutting device to ensure parallel surfaces, with the resulting constructs ranging in thickness from 1.0–1.3 mm.

LOADING PROTOCOLS

Intermittent dynamic loading (DL) was carried out in a custom deformational loading bioreactor[25] in a volume of 5 ml DMEM. The loading regime employed consisted of ~10% deformation, at 1 Hz, with three cycles of 1 h on/1 h off over a period of 6 h, resulting in a total of 3 h loading/day, for 5 days/week. Free-swelling (FS) controls were maintained in the same amount of medium adjacent to the loading device during loading periods. After loading, disks were returned to 30 ml of fresh medium for overnight culture. Every two weeks, 3–4 disks were removed for analysis over an eight-week period.

MECHANICAL TESTING

Mechanical testing was carried out in unconfined compression between impermeable platens in a custom mechanical testing device[33]. Constructs were first equilibrated in creep under a tare load of ~0.02 N, followed by stress relaxation tests with a ramp displacement of 1 µm/second to 10% strain (based on the measured post-creep thickness). After equilibrium was reached (2000 seconds), a sinusoidal displacement of 40 µm amplitude was applied at frequencies ranging from 0.005–1 Hz. The compressive Young's modulus was determined from the equilibrium response of the stress-relaxation test by dividing the equilibrium stress (minus the tare stress) by the applied strain. Dynamic moduli at each testing frequency were calculated from the ratio of the measured stress amplitude and the applied strain amplitude of dynamic loading. Articular cartilage specimens (with underlying bone removed) were tested similarly, with a creep load of ~0.1 N, and a dynamic displacement of 20 µm amplitude.

BIOCHEMICAL COMPOSITION

Biochemical properties of articular cartilage samples and engineered constructs were assessed as described previously[25,26,32]. Constructs and articular cartilage samples were weighted wet, lyophilized, reweighted dry, and digested with papain at 60°C for 18 h. Glycosaminoglycan (GAG) content, reported as percent wet weight of the tissue (% ww) was assessed using the DMMB assay[34,35], scaled for microplates. Collagen content, as assessed by measurement of orthohydroxyproline (OHP), was measured via a colorimetric reaction of a sample of acid hydrolyzed digest with diethyamimobenzaldehyde and chloramine T[36]. A collagen:OHP ratio of 10:1 was used as a conversion factor[22].

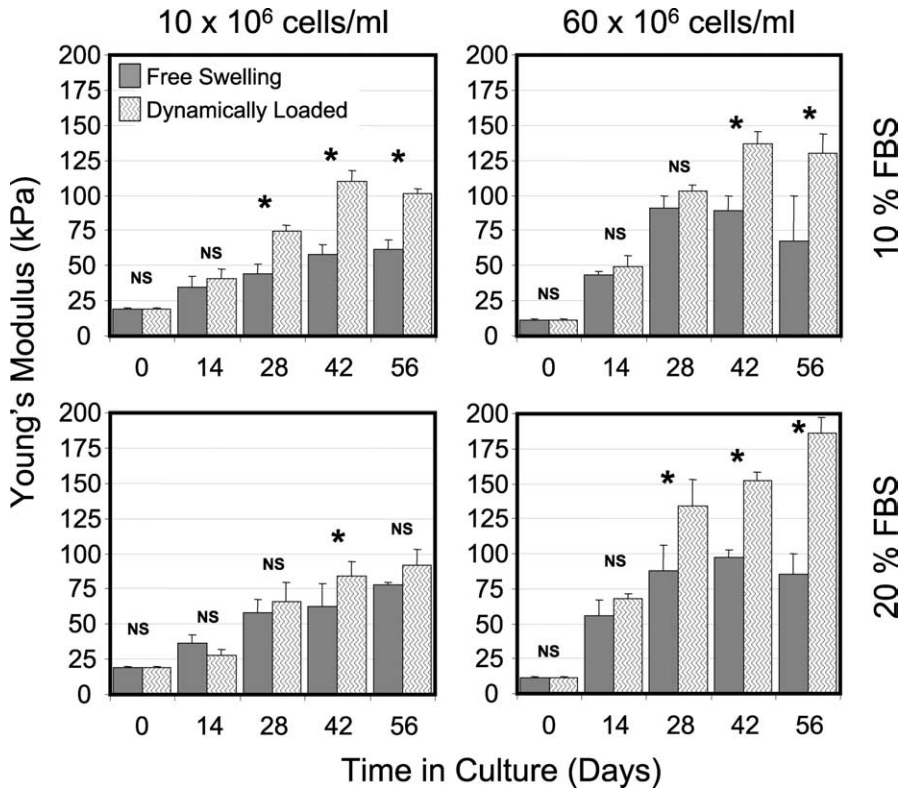

Fig. 1. Equilibrium Young's modulus (E_Y) with time in culture for constructs seeded at 10 and 60×10^6 cells/ml cultured in either 10% or 20% FBS containing medium. Data represent the mean and standard deviation of 3–4 samples. NS: not significant (P>0.05), *indicates significant difference from free-swelling control at same time point and culture condition (P<0.05).

HISTOLOGY

Samples for histology were fixed overnight at 4°C in acid/formalin/ethanol, and dehydrated in a graded series of ethanol. Samples were then embedded in paraffin, sectioned to 8 μm, and affixed to microscope slides. Sections were then stained with Hematoxylin and Eosin, Safranin O, and Picrosirius Red/Alcian Blue, as described previously[32]. Stained sections were photographed using a dissecting microscope (Olympus, Japan). Scale bars are provided for each image.

STATISTICS

Statistics were performed using one- and two-way ANOVA with Fisher's LSD post-hoc tests, with α=0.05. All data are reported as the mean ± standard deviation of 3–4 samples per time point

Results

NATIVE TISSUE PROPERTIES: BOVINE CMC CARTILAGE

Bovine carpometacarpal cartilage from which the cells were isolated was analyzed for material properties, biochemical composition, and histological features using the same protocol as for engineered constructs. The measured Young's modulus of the native tissue was 277±83 kPa (N=5). Dynamic modulus was found to vary with loading frequency, increasing from 2.8±0.8 MPa at a frequency of 0.005 Hz to 7.0±0.7 MPa at a frequency of 1.0 Hz (P<0.001). Biochemically, the GAG content of bovine CMC cartilage was 2.4±0.5% ww while the collagen content was 21.5±2.7% ww. Staining with Safranin O and Picrosirus Red/Alcian Blue revealed a rich red staining spanning the extent of the tissue.

MECHANICAL PROPERTIES OF ENGINEERED CONSTRUCTS

Time, loading condition (Free-Swelling vs Dynamic Loading), initial cell seeding density, and percentage FBS all had a significant effect on the Young's modulus of chondrocyte-seeded agarose constructs (P<0.001). The interaction of all of these terms was also significant (P<0.05).

Constructs seeded at 10 million cells/ml and cultured in 10% FBS yielded constructs with increasing material properties with time in culture (Fig. 1, top left). By day 28, the Young's modulus of dynamically loaded constructs was significantly higher than free-swelling constructs (P<0.001) and continued to be so through day 56 (P<0.001). Neither free-swelling controls nor dynamically loaded samples changed significantly over the last two weeks of culture (P=0.72 and P=0.29, respectively). On day 56, free-swelling constructs had a Young's modulus of 61.3± 7.5 kPa, while constructs cultured with dynamic deformational loading reached 101.5±3.5 kPa. When these constructs were cultured in DMEM supplemented with 20% FBS (Fig. 1, bottom left), a different response was observed. Dynamically loaded constructs were generally similar to free-swelling controls, except for a small but significant increase in dynamically loaded constructs on

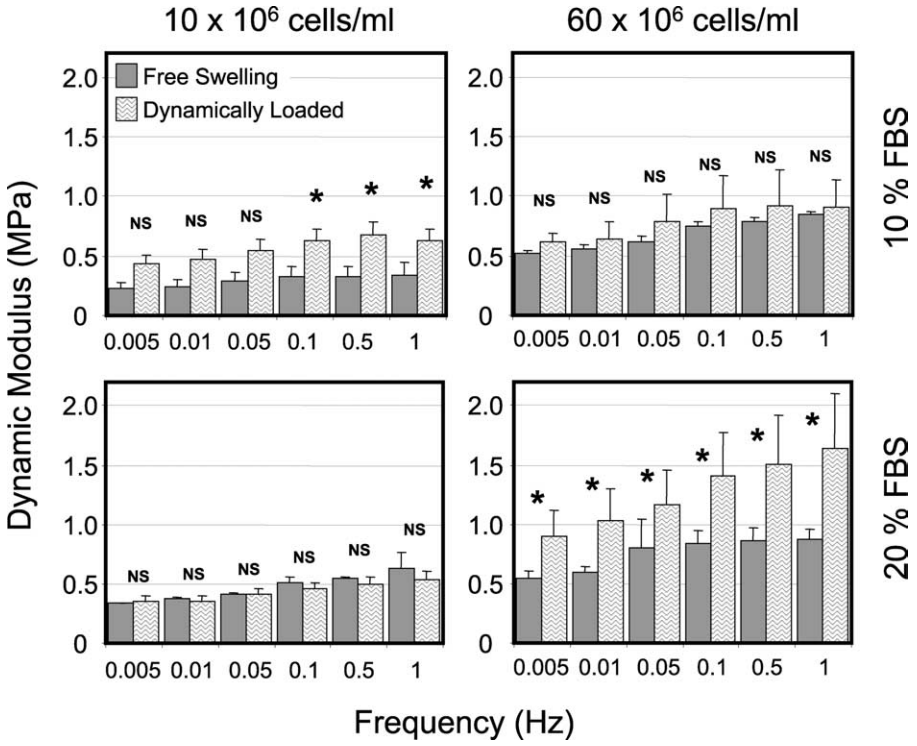

Fig. 2. Dynamic modulus at frequencies ranging from 0.005–1 Hz for constructs on day 56 seeded at 10 and 60×10^6 cells/ml cultured in either 10% or 20% FBS containing medium. Data represent the mean and standard deviation of 3–4 samples. NS: not significant (P>0.05), *indicates significant difference from free-swelling control at same frequency and culture condition (P<0.05).

day 42 (P<0.005). On day 56, free-swelling constructs reached 78.0±1.5 kPa, while dynamically loaded constructs had a Young's modulus of 91.5±11.6 kPa (NS vs free-swelling, P=0.27). Between days 42 and 56 there was no significant change in either free-swelling or dynamically loaded samples (P=0.06 and P=0.44, respectively).

For cultures initially seeded with 60 million cells per ml cultured in 10% FBS, dynamic deformational loading was observed to have no effect until day 42, at which point loaded constructs were significantly stiffer than free-swelling controls (P<0.001, Fig. 1, top right). Loaded constructs continued to be significantly stiffer than free-swelling controls on day 56, when free-swelling controls reached a Young's modulus of 67.8±31.7 kPa while dynamically loaded samples reached 129.6±14.4 kPa (P<0.001, Free-Swelling vs Dynamically Loaded, day 56). Free-swelling constructs showed a decrease in stiffness (P<0.005) while dynamically loaded constructs showed no change (P=0.38) between days 42 and 56. When these constructs were cultured in 20% FBS (Fig. 1, bottom right), similar trends were found. Dynamically loaded samples became significantly higher than free-swelling controls by day 28 (P<0.001). This increase with dynamic loading continued to be significant through days 42 (P<0.001) and 56 (P<0.001). On day 56, free-swelling constructs reached a Young's modulus of 85.1±15.0 kPa while dynamically loaded samples reached 186.2±11.3 kPa, the highest value achieved in this study. Between days 42 and 56, free-swelling sample showed no significant change (P=0.12) while dynamically loaded constructs continued to increase in stiffness (P<0.0001).

On day 56, neither seeding density (10 or 60 million cells per ml) nor percentage FBS (10 or 20%) had a significant effect on free-swelling controls. However, free-swelling constructs seeded at 60 million cells cultured in 20% FBS were significantly higher than those seeded at 10 million cells per ml and maintained in 10% FBS containing medium. For dynamically loaded samples, samples at 60 million cells per ml had a higher Young's modulus than similarly maintained constructs seeded at 10 million cells per ml (P<0.002). Increasing medium FBS concentration from 10% to 20% had no effect on dynamically loaded samples seeded at 10 million cells per ml (P=0.26), but significantly increased the Young's modulus of dynamically loaded samples seeded at 60 million cells per ml (P<0.001). The Young's modulus of dynamically loaded constructs at 60 million cells per ml maintained in 20% FBS containing medium was significantly higher than all other constructs at either seeding density (P<0.001).

Long-term culture also revealed significant differences in the dynamic modulus of chondrocyte-seeded agarose constructs, dependent on cell seeding density, FBS concentration, dynamic loading, and the frequency at which the modulus was measured (P<0.001 for all). Seeding density, concentration of FBS, and long-term deformational loading were also seen to have an interactive influence on the measured dynamic modulus (p<0.001). The dynamic modulus on day 56 for constructs for all seeding and culture conditions at each frequency are presented in Fig. 2.

For constructs seeded at 10 million cells per ml cultured in 10% FBS (Fig. 2, top left), long-term dynamic loading had no effect on dynamic modulus at frequencies of 0.005, 0.01, and 0.05 Hz (P=0.13, P=0.08, P=0.06, respectively), while there was a significant increase in dynamic modulus for testing frequencies of 0.1, 0.5, and 1.0 Hz (P<0.05 for all). The dynamic modulus was not found to change with

increasing testing frequency. At a testing frequency of 1 Hz, free-swelling samples had a dynamic modulus of 0.34± 0.11 MPa, while dynamically loaded constructs had a dynamic modulus of 0.63±0.10 MPa (P<0.05). Culture of 10 million cells per ml constructs in 20% FBS containing medium (Fig. 2, bottom left) resulted in no change with deformational loading at any frequency (P>0.5). Increasing testing frequency did not change the measured dynamic stiffness of either free-swelling or constructs cultured with dynamic deformational loading. At a testing frequency of 1 Hz, free-swelling samples had a dynamic modulus of 0.63±0.13 MPa, while dynamically loaded constructs had a dynamic modulus 0.54±0.07 MPa (NS, P=0.53).

At the higher seeding density, 60 million cells per ml, the measured dynamic modulus was seen to depend on the concentration of FBS in the culture medium. For constructs cultured in medium containing 10% FBS (Fig. 2, top right), free-swelling samples were not significantly different from dynamically loaded samples at any frequency (P>0.3). In this case, however, the dynamic modulus of free-swelling constructs measured at 1 Hz was significantly higher than that measured at a frequency of 0.005 Hz (P<0.001). At a testing frequency of 1 Hz, free-swelling samples had a dynamic modulus of 0.85±0.02 MPa, while dynamically loaded constructs had a dynamic modulus 0.91±0.22 MPa (NS, P=0.70). When medium was supplemented with 20% FBS (Fig. 2, bottom right), dynamic loading was found to have a significant effect on the measured dynamic modulus at every testing frequency (P<0.025). Furthermore, the dynamic modulus of free-swelling samples tested at 1 Hz was significantly higher than that measured at both 0.01 (P<0.05) and 0.005 Hz (P<0.025). The dynamic modulus of dynamically loaded constructs tested at 1 Hz was significantly higher than that measured at 0.005 (P<0.001), 0.01 (P<0.001), and 0.05 (P<0.001) Hz. At a testing frequency of 1 Hz, free-swelling samples had a dynamic modulus of 0.87±0.09 MPa, while dynamically loaded constructs had a dynamic modulus 1.64±0.46 MPa (P<0.001).

Overall, free-swelling constructs showed no significant change in the measured dynamic modulus (at 1 Hz) with increasing FBS concentration (P=0.06, 10 million cells per ml; P=0.87, 60 million cells per ml). Increasing cell seeding density (from 10 to 60 million cell per ml) significantly increased free-swelling constructs at 10% FBS supplementation (P<0.005), but not at 20% FBS supplementation (NS, P=0.12). For dynamically loaded samples, increasing FBS concentration had no effect on 10 million cells per ml constructs (P=0.51), while leading to significant increases for 60 million cells per ml constructs (P<0.001). Increasing cell number at a given FBS concentration was not significant at 10% FBS (P=0.07), but was significant at 20% FBS (P<0.001). The dynamic modulus of dynamically loaded constructs seeded at 60 million cells per ml in 20% FBS containing medium was significantly higher than all other constructs (P<0.001).

BIOCHEMICAL COMPOSITION OF ENGINEERED CONSTRUCTS

In this study the GAG content of chondrocyte-seeded agarose constructs was found to depend on the time in culture (P<0.001) and initial cell seeding density (P<0.001), but not on loading condition (P=0.87) or FBS supplementation (P=0.17).

Constructs seeded at 10 million cells per ml and cultured in 10% FBS (Fig. 3, top left) with dynamic deformational loading contained more GAG than free-swelling controls by day 42 (P<0.01). At this time point, free-swelling controls contained 0.99±0.07% ww GAG, while dynamically loaded samples contained 1.67±0.28% ww GAG. Over the last two weeks of culture, GAG content remained constant for free-swelling samples (P=0.15), while dynamically loaded samples continued to increase (P<0.001). When these constructs were cultured in 20% FBS (Fig. 3, bottom left), GAG content did not depend on dynamic deformational loading at any time point (P>0.40). Furthermore, GAG content did not change for either loading condition over the last two weeks (P>0.25). On day 42, free-swelling construct contained 1.27±0.21% ww GAG and dynamically loaded samples contained 1.06±0.25% ww GAG.

For constructs initially seeded at 60 million cells per ml cultured in 10% FBS containing medium (Fig. 3, top right) dynamic loading did not influence the GAG content of constructs (P>0.37). On day 42, free-swelling constructs contained 1.59±0.07% ww GAG, while dynamically loaded constructs contained 1.69±0.73% ww GAG (P=0.67, Free Swelling vs Dynamically Loaded, Day 42). When these higher density constructs were cultured in medium supplemented with 20% FBS, a similar finding was observed (Fig. 3, bottom right). At no time in culture did dynamic deformational loading have an effect on the GAG content (P>0.23). On day 42, free-swelling samples reached 1.89±0.47% ww GAG, while dynamically loaded samples reached 1.74±0.37% ww GAG (P=0.55, Free-Swelling vs Dynamically Loaded, day 42).

On day 42, for free-swelling samples, increases in seeding density increased GAG content (P<0.025 in 10% FBS, P<0.025 in 20% FBS), while increases in FBS concentration did not (P=0.26 for 10 million cells per ml and P=0.22 for 60 million cells per ml). For dynamically loaded samples, increases from 10 to 60 million cells per ml increased GAG content in 20% FBS (P<0.01), but not in 10% FBS (P=0.42) containing medium. Changing serum concentration led to significant differences in GAG content in 10 million cell per ml constructs (P<0.025), but not in 60 million cell per ml constructs.

Collagen content was also observed to depend on time in culture (P<0.001), FBS concentration (P<0.001), and initial cell seeding density (P<0.001), but not on long term deformational loading (NS, P=0.82).

For constructs seeded at 10 million cells per ml cultured in 10% FBS (Fig. 4, top left), dynamic loading led to increases in collagen content relative to free-swelling controls on days 28 (P<0.005), 42 (P<0.025), and 56 (P<0.025). On day 56, free-swelling samples contained 2.11±0.65% ww collagen, while dynamically loaded samples contained 2.68±0.31% ww collagen. Increasing the FBS concentration to 20% (Fig. 4, bottom left) resulted in a construct collagen content that was not different between loading conditions after day 14 (P>0.18). On day 56, free-swelling samples contained 2.24±0.25% ww collagen, while dynamically loaded samples contained 2.18±0.04% ww collagen (NS, P=0.78, Free-Swelling vs Dynamically Loaded, Day 56).

Increasing initial seeding density to 60 million cells per ml, with culture in 10% FBS containing medium (Fig. 4, top right) resulted in constructs where dynamic loading had no influence on collagen content at any time of culture (P>0.22). On day 56, collagen content of constructs reached 3.90±0.52% ww collagen in free-swelling conditions and 3.64±0.40% ww in dynamically loaded conditions. At this seeding density, culture in 20% FBS containing medium (Fig. 4, bottom right) resulted in constructs in which dynamic loading had no effect on collagen content at any time in culture (P>0.10).

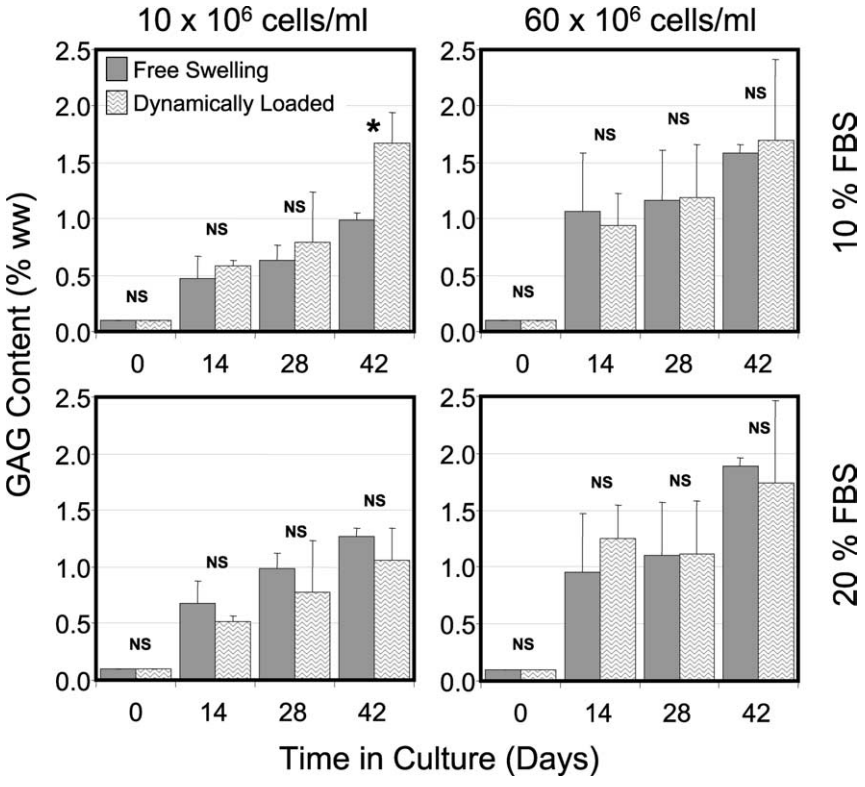

Fig. 3. Percent glycosaminoglycan (GAG) content (of wet weight [ww]) with time in culture for constructs seeded at 10 and 60×10^6 cells/ml cultured in either 10% or 20% FBS containing medium. Data represent the mean and standard deviation of 3-4 samples. NS: not significant (P>0.05), *indicates significant difference from free-swelling control at same time point and culture condition (P<0.05).

On day 56, free-swelling constructs reached 3.00± 0.13% ww collagen while dynamically loaded constructs reached 2.64±0.54% ww (NS, P=0.10, Free-Swelling vs Dynamically Loaded, day 56).

Free-swelling cultures, on day 56, showed significant increases on collagen content with increasing seeding density at both 10 (P<0.001) and 20% (P<0.001) FBS. Increasing FBS concentration at 10 million cells per ml did not affect collagen content (P=0.58), while the same increase in FBS led to decreases in collagen content at 60 million cells per ml (P<0.001). Dynamically loaded constructs, at day 56, showed increased collagen content as a function of both seeding density (P<0.05) and increasing FBS concentration (P<0.025). At the end of culture, both free-swelling and dynamically loaded constructs seeded at 60 million cells per ml cultured in 10% FBS were higher than all other seeding and culture conditions (P<0.001).

PHYSICAL AND HISTOLOGICAL FEATURES OF ENGINEERED CONSTRUCTS

With time in culture, constructs became increasingly opaque (Fig. 5), and changed in thickness depending on the culture conditions. When constructs were cultured in 20% FBS, bulbous cells growths were occasionally observed at the periphery of constructs, especially in free-swelling constructs (Fig. 5, arrows). On day 0, constructs seeded with 10 and 60 million cells per ml showed little staining for proteoglycans (Fig. 6A, left most images). By day 42, a clear increase in proteoglycan content, as assessed by Safranin O staining, was observed in all conditions (Fig. 6A). In some cases, proteoglycan staining was more intense in loaded constructs compared to free-swelling controls (i.e, for 10 million cells/ml, 10% FBS), confirming bulk measurements of GAG. Collagen content, as assessed by Picrosirius Red staining, increased with time in culture and seeding density. Collagen deposition (pink color) was observed to increase with deformational loading in 10 million cell per ml constructs in 10% FBS supplemented medium but showed no clear differences between free-swelling and dynamically loaded constructs for any other culture condition (Fig. 6B).

Disk thickness was found to depend on time in culture, initial cell seeding density, FBS concentration, and loading condition (Fig. 7, P<0.001). The interactive effect of these stimuli was also significant (P<0.001). For all constructs, dynamic loading resulted in constructs with less axial swelling than observed for free-swelling controls by day 56 (P<0.005). This effect was observed at increasingly earlier times in culture with increases in FBS concentration and seeding density.

Discussion

The current study explores the enhancement of matrix elaboration and material properties of chondrocyte-seeded agarose hydrogel constructs with the application of daily dynamic deformational loading over a long-term culture period. In the present study, an increased volume of medium was employed so as to initiate cultures with less than 1.7 million cells maintained per milliliter of feed

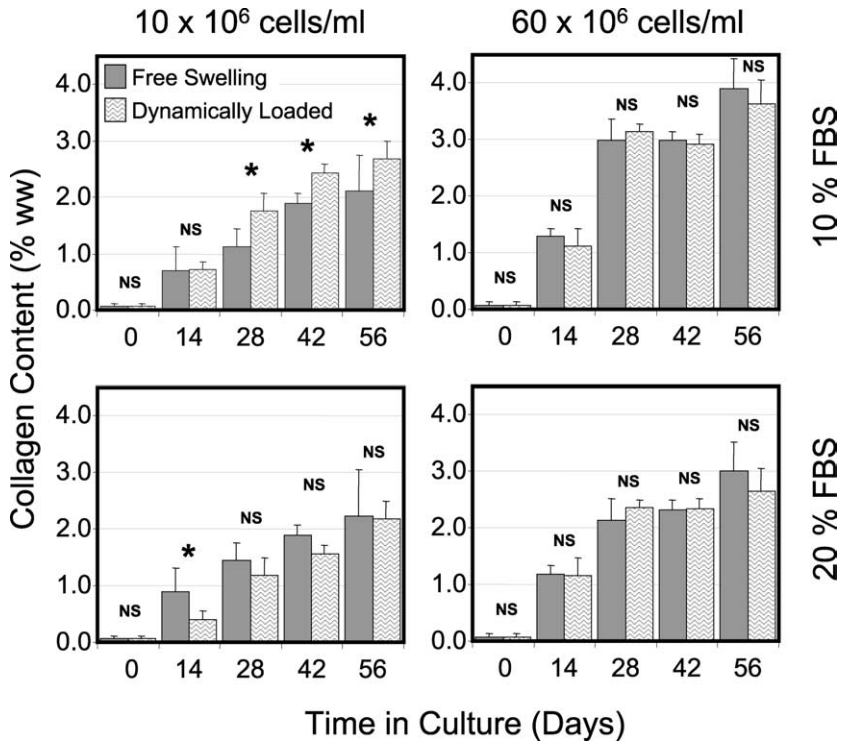

Fig. 4. Percent collagen content (of the wet weight [ww]) with time in culture for constructs seeded at 10 and 60×10^6 cells/ml cultured in either 10% or 20% FBS containing medium. Data represent the mean and standard deviation of 3–4 samples. NS: not significant (P>0.05), *indicates significant difference from free-swelling control at same time point and culture condition (P<0.05).

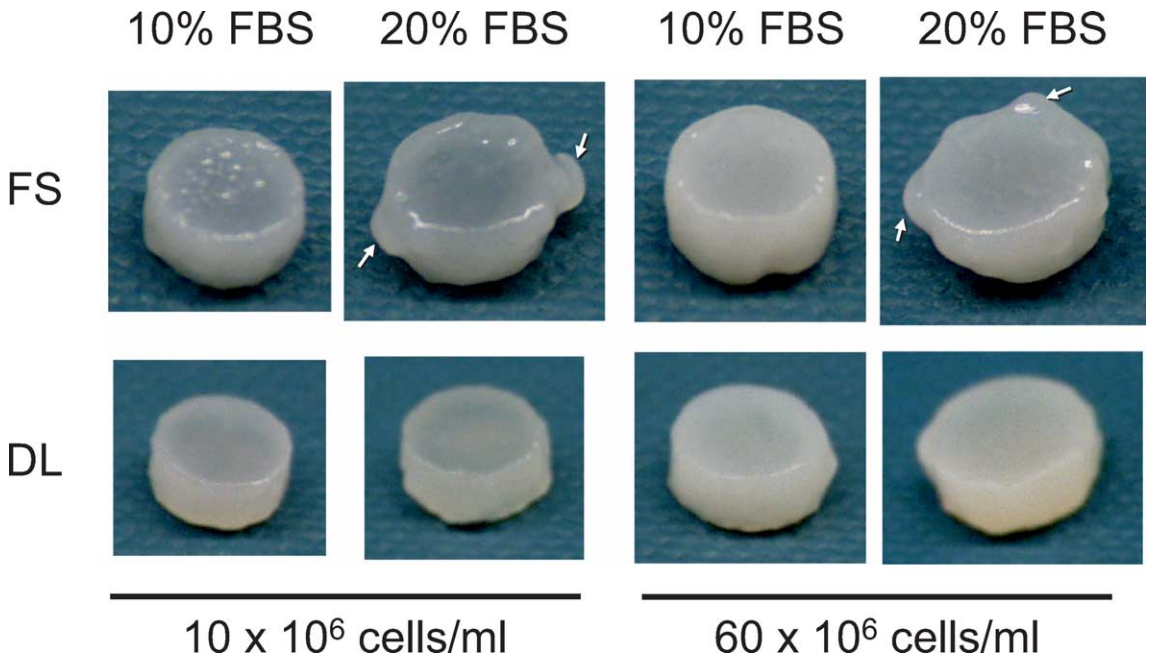

Fig. 5. Gross appearance of constructs on day 42. Arrows indicate cell masses forming on the periphery of some constructs.

medium/day (in comparison to as much as 3.5 million cells/ml feed medium/day in our previous studies[26]). Under these new culture conditions, we explored the effect of initial cell seeding density (10 or 60 million cells/ml) and serum concentration (10% FBS or 20% FBS) on the ability of applied deformational loading to promote functional tissue growth *in vitro.* The results of this study confirm our initial hypothesis, demonstrating that the effects of deformational loading were most pronounced at the higher cell seeding density studied (60 million cells/ml) when cultured in the highest concentration of fetal bovine serum (20%).

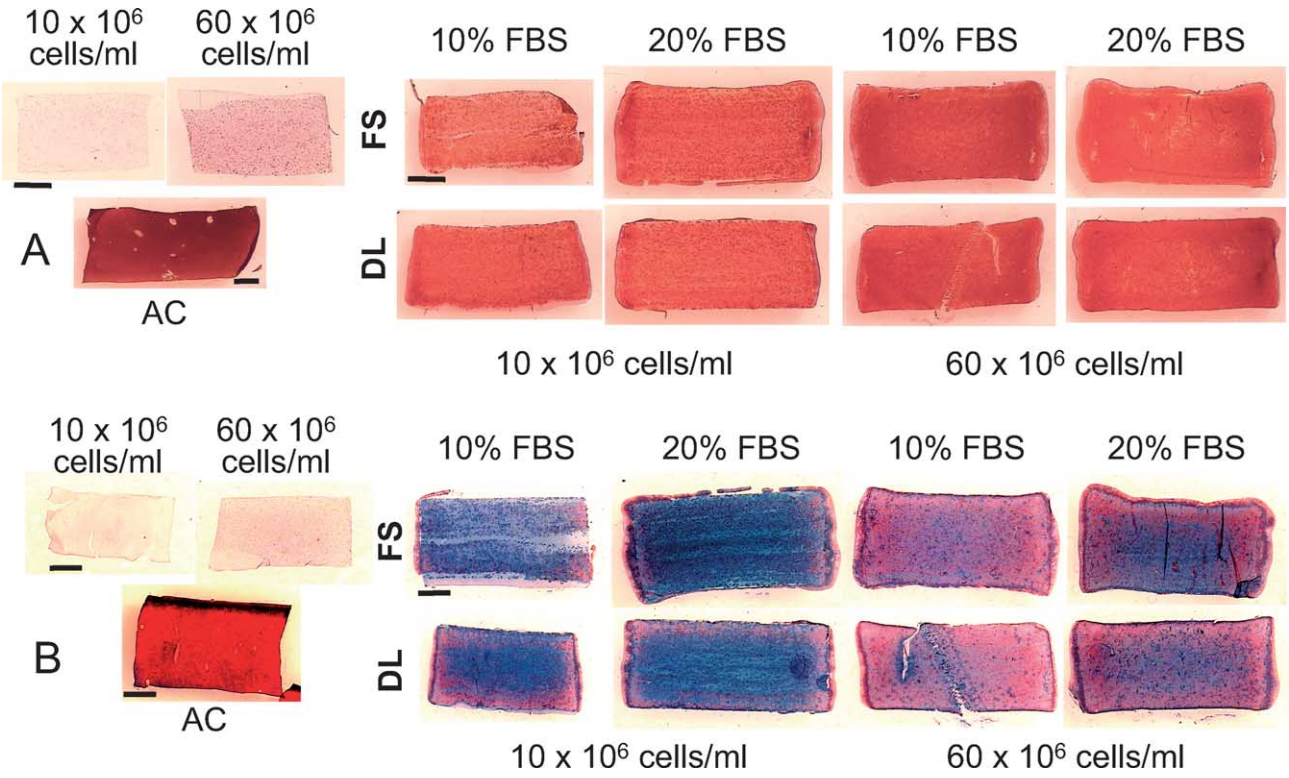

Fig. 6. A: Cross sectional views of Safranin O stained sections of articular cartilage (AC, bottom left) and engineered constructs on day 0 (left most images) and day 42 (right most images) under free-swelling (FS) and dynamically loaded (DL) conditions. B: Cross sectional views of Picrosirius Red/Alcian Blue stained sections of articular cartilage (AC, bottom left) and engineered constructs on day 0 (left most images) and day 42 (right most images) under free-swelling (FS) and dynamically loaded (DL) conditions. Scale bar equals 1 mm for all cross sections.

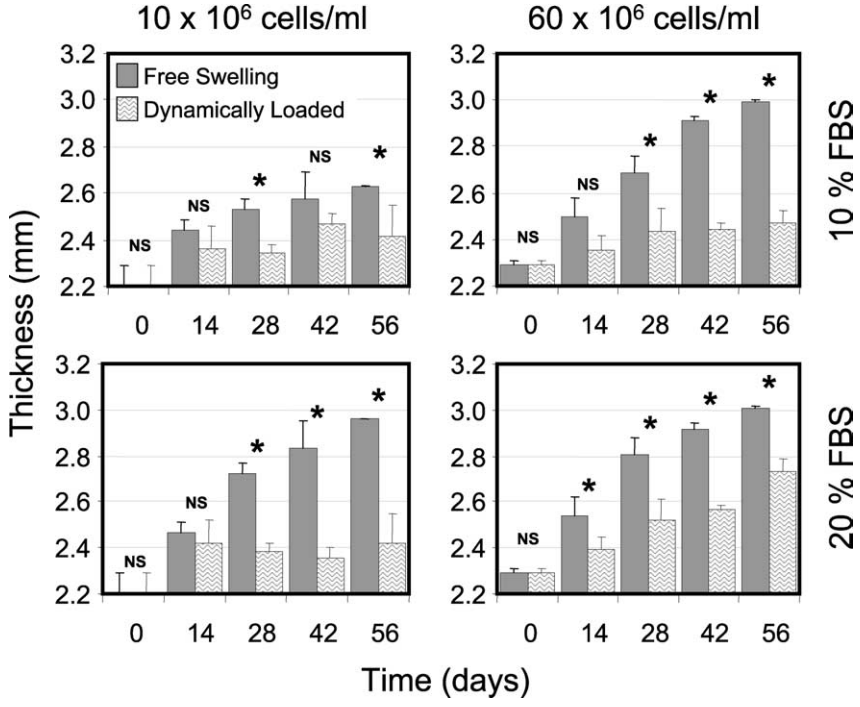

Fig. 7. Thickness with time in culture for constructs seeded at 10 and 60×10⁶ cells/ml cultured in either 10% or 20% FBS containing medium. Data represent the mean and standard deviation of 3–4 samples. NS: not significant (P>0.05), *indicates significant difference from free-swelling control at same time point and culture condition (P<0.05).

In this study, constructs achieved a higher magnitude of stiffness (to ~180 kPa) than in our previous studies, with an ~2-fold increase in both the Young's and dynamic moduli of dynamically loaded constructs (on day 56) compared to free-swelling controls. The relative effect of loading is less than observed previously (where we have generally found a >3-fold enhancement over free-swelling controls[25,26]). This is due to the substantially better performance (e.g., increase in stiffness) of the free-swelling control group observed in this study compared to that previously observed[25,26]. In contrast to the current study, our previous study at 60 million cells/ml showed no effect of deformational loading[26]. Considering that twice the number of cells were maintained in the same amount of feed medium in this previous study, we speculate that this lack of response is most likely attributed to nutrient insufficiency. In this regard, the most critical change from our original protocol was the decrease in the number of cells maintained in a set amount of feed medium adopted in the current study. Even with this increase in nutrient supply, however, the increase in construct mechanical properties in response to dynamic deformational loading emerged only at later times in culture with the higher seeding density studied, and was more robust in the presence of 20% FBS. Thus the increase in nutrient supply employed in this study provides an environment that is permissive for construct growth and response to deformational loading at higher seeding densities over a two month time period.

The effect of nutrient supply appears to be graded depending on the cell seeding density of the constructs. In a nutrient-poor environment, free-swelling constructs increase in stiffness through a certain period of culture, and thereafter their properties plateau, or even decrease[26]. In this study, for example, constructs seeded at 10 million cells/ml cultured in 10% FBS containing medium exhibited a steady increase in Young's modulus whereas 60 million cells/ml constructs in 10% FBS increased quickly, plateaued, and declined. Interestingly, this effect is apparent despite the fact that the number of cells per volume of feed medium per day begins at <1.7 million cells/ml feed medium/day and then decreases gradually through later times in culture, as 3–4 disks per time point are removed for analysis. While not measured in this study, in similar studies the cell number/DNA content of agarose constructs seeded at 60 million cells/ml was found to change by less than 15% over a 42 day culture period[26,37]. This suggests that rather than changes in cell number, either a limited nutrient supply during early time periods leads to permanent deleterious effects or that as matrix accumulates an increasingly dense barrier to diffusion arises in engineered constructs.

A number of studies have explored the effect of serum concentration on the growth of tissue-engineered constructs, with higher levels of FBS generally leading to increased growth[38]. Fetal bovine serum (FBS) contains a number of molecules that are necessary for mammalian cell growth, including growth factors, trace elements, lipids, polyamines, and transport proteins[39]. The beneficial effects of deformational loading observed in the current study may arise from the enhanced inwardly directed convective transport of large molecules found in serum (like growth factors). This phenomenon has been observed experimentally in dynamically loaded cartilage explants[40,41] and can be theoretically predicted using a recently developed model of neutral solute transport in dynamically loaded porous permeable gels[42]. At a lower cell seeding density, increases in serum concentration appeared to mask the effects of dynamic loading. Specifically, loading has a positive effect on 10 million cells/ml constructs maintained in 10% FBS containing medium, which was not evident with 20% FBS. At this lower seeding density, 20% FBS may be saturating with respect to the availability of soluble factors and the maximal biosynthetic activity of a given number of cells. For the 60 million cells/ml group, 10% FBS did not appear to be sufficient to maintain positive growth of constructs in free-swelling culture, as evident from the initial rise and then decrease of material properties over the last two weeks of the 56 day culture period. Deformational loading appeared to counteract the decreases observed in free-swelling conditions, though loaded constructs did plateau during later culture times (between day 42 and 56). From a mechanistic point of view, it is difficult to determine if the increased nutrient supply acts to provide soluble factors that lead directly to enhanced matrix elaboration (e.g., nutrient transport) or indirectly through modulation of the mechanosensitivity of the chondrocytes, or some combination. Such mechano-chemical coupling has been observed in a variety of basic science studies[40,43,44] and bioreactor systems[16,30,45].

As chondrocytes embedded in agarose secrete extracellular matrix molecules, they become entrapped in the polysaccharide chains of the hydrogel and coalesce into a functional matrix[8,9]. Agarose (and other hydrogels) may be particularly suitable in this regard, as it traps these matrix molecules to a greater extent than other fibrous scaffold systems (which release products to the medium)[46]. With the accumulation of these molecules, both loaded and free-swelling disks become increasingly opaque over the culture period. In this study, despite the fact that the bulk content of GAG and collagen was found to be similar between loaded and free-swelling constructs seeded at 60 million cells/ml, the former exhibited significantly greater material properties. One possible explanation for this finding is that while the amount of sulfated GAG molecules is similar in free-swelling and dynamically loaded constructs, the assembly of these molecules into large macromolecular aggregates is regulated by deformational loading (either intrinsically[47] or via small molecules such as link protein[48]). For example, static compression has been demonstrated to differentially regulate the synthesis of link protein, aggrecan, and hyaluronan in cartilage explants[49]. In a tissue-engineering context, studies using an alginate hydrogel have demonstrated that static deformational loading increased the amount of GAG lost to the medium without altering the structure of the aggrecan core protein[50]. In addition to the size of aggregates and organization of the proteoglycan network, other extracellular matrix components (e.g., type IX collagen[51], and cartilage oligomeric matrix protein (COMP)[52,53]), which participate in the cross-linking of the type II collagen extracellular matrix, may be differentially regulated with deformational loading and thereby influence the functional properties of the ECM. Indeed, recent studies have shown that COMP gene expression is elevated in explants and alginate disks in response to deformational loading[54,55]. As a final possibility, Quinn *et al.* (2002)[56] recently reported (using a radionucleotide tracer technique) that up to 14 days are necessary for formed proteoglycan molecules to come in contact with one another in a free-swelling agarose hydrogel system. The movement of these molecules as well as other large matrix components may be accelerated by deformational loading, decreasing the time necessary for their functional interaction and increasing the range over which these interactions occur in the developing construct.

This study confirms that deformational loading under the proper conditions enhances the mechanical properties of chondrocyte seeded agarose hydrogels. We further show that increases in seeding density and serum concentration can significantly enhance this *in vitro* growth. In eight weeks, the Young's modulus of engineered constructs approached ~3/4 that of native young bovine tissue. Over this same period, the dynamic unconfined modulus reached ~1/4 the value of the native tissue, and began to show the frequency dependent response characteristic of the native tissue. The dynamic modulus in unconfined compression is particularly important measure of how an engineered construct will perform in an *in vivo* dynamic loading environment, and gives some insight into the emerging tensile properties of the tissue[57]. The measured mechanical properties of engineered constructs in this study compare favorably with constructs seeded at nearly twice the seeding density in PGA felts grown *in vitro* in rotating wall bioreactors for 6–12 weeks[14,21,22]. In addition to mechanical properties, the biochemical constituents of engineered constructs reached peak values of ~2/3 native tissue levels for GAG and ~1/5 that of native tissue for collagen. In most cases, however, bulk measures of collagen and GAG were not found to correlate with the mechanical properties of engineered constructs. This finding suggests that traditional bulk measures used in cartilage tissue engineering studies represent only an initial evaluation of construct growth, and necessitate further examination of the bulk and local mechanical properties and the functional nature of the deposited extracellular matrix[4,5,58]. In this study deformational loading was also found to maintain construct shape, with significantly less axial swelling in loaded constructs compared to free-swelling controls, particularly at higher seeding densities and serum concentrations. This finding may be particularly important for the production of anatomically shaped engineered cartilage constructs[11,59,60]. Taken together, these findings represent a significant step forward in the definition of the culture conditions that will facilitate the functional tissue engineering of articular cartilage.

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References

1. Ateshian GA, Hung CT. Functional properties of native articular cartilage. In: Guilak F, Butler DL, Goldstein SA, Mooney DJ, Eds. Functional tissue engineering. New York: Springer-Verlag 2003;46–68.
2. Schinagl RM, Gurskis D, Chen AC, Sah RL. Depth-dependent confined compression modulus of full-thickness bovine articular cartilage. J Orthop Res 1997;15(4):499–506.
3. Williamson AK, Chen AC, Sah RL. Compressive properties and function-composition relationships of developing bovine articular cartilage. J Orthop Res 2001;19(6):1113–21.
4. Wang CCB, Deng JM, Ateshian GA, Hung CT. An automated approach for direct measurement of two-dimensional strain distributions within articular cartilage under unconfined compression. J Biomech Eng 2002;124(5):557–67.
5. Wang CCB, Chahine NO, Hung CT, Ateshian GA. Optical determination of anisotropic properties of bovine articular cartilage in compression. J Biomech 2000; 36(3):339–53.
6. Muir H. The chemistry of the ground substance of joint cartilage. In: Sokoloff L, Ed. The Joints and Synovial Fluid. New York: Academic Press 1980;II:27–94.
7. Hunziker EB. Articular cartilage repair: are the intrinsic biological constraints undermining this process insuperable? Osteoarthritis Cartilage 1999;7:15–28.
8. Buschmann MD, Gluzband YA, Grodzinsky AJ, Kimura JH, Hunziker EB. Chondrocytes in agarose culture synthesize a mechanically functional extracellular matrix. J Orthop Res 1992;10(6):745–58.
9. Buschmann MD, Gluzband YA, Grodzinsky AJ, Hunziker EB. Mechanical compression modulates matrix biosynthesis in chondrocyte/agarose culture. J Cell Sci 1995;108(Pt 4):1497–508.
10. Carver SE, Heath CA. Increasing extracellular matrix production in regenerating cartilage with intermittent physiological pressure. Biotechnol Bioeng 1999;62(2):166–74.
11. Chang SC, Rowley JA, Tobias G, *et al.* Injection molding of chondrocyte/alginate constructs in the shape of facial implants. J Biomed Mater Res 2001; 55(4):503–11.
12. Dunkelman NS, Zimber MP, LeBaron RG, Pavelec R, Kwan M, Purchio AF. Cartilage production by rabbit articular chondrocytes on polyglycolic acid scaffolds in a closed bioreactor system. Biotech Bioeng 1995; 46:299–305.
13. Freed LE, Hollander AP, Martin I, Barry JR, Langer R, Vunjak-Novakovic G. Chondrogenesis in a cell-polymer-bioreactor system. Exp Cell Res. 1998; 240(1):58–65.
14. Freed LE, Langer R, Martin I, Pellis NR, Vunjak-Novakovic G. Tissue engineering of cartilage in space. Proc Natl Acad Sci USA 1997;94(25): 13885–90.
15. Freed LE, Vunjak-Novakovic G, Langer R. Cultivation of cell-polymer cartilage implants in bioreactors. J Cell Biochem 1993;51(3):257–64.
16. Gooch KJ, Blunk T, Courter DL, *et al.* IGF-I and mechanical environment interact to modulate engineered cartilage development. Biochem Biophys Res Commun 2001;286(5):909–15.
17. Gooch KJ, Kwon JH, Blunk T, Langer R, Freed LE, Vunjak-Novakovic G. Effects of mixing intensity on tissue-engineered cartilage. Biotechnol Bioeng 2001; 72(4):402–7.
18. Obradovic B, Carrier RL, Vunjak-Novakovic G, Freed LE. Gas exchange is essential for bioreactor cultivation of tissue engineered cartilage. Biotechnol Bioeng 1999;63(2):197–205.
19. Pazzano D, Mercier KA, Moran JM, *et al.* Comparison of chondrogensis in static and perfused bioreactor culture. Biotechnol Prog 2000;16(5):893–6.
20. Puelacher WC, Kim SW, Vacanti JP, Schloo B, Mooney D, Vacanti CA. Tissue-engineered growth of cartilage: the effect of varying the concentration of chondrocytes seeded onto synthetic polymer matrices. Int J Oral Maxillofac Surg 1994;23(1): 49–53.

21. Martin I, Obradovic B, Treppo S, *et al.* Modulation of the mechanical properties of tissue engineered cartilage. Biorheology 2000;37(1-2):141–7.
22. Vunjak-Novakovic G, Martin I, Obradovic B, *et al.* Bioreactor cultivation conditions modulate the composition and mechanical properties of tissue-engineered cartilage. J Orthop Res 1999;17(1): 130–8.
23. Butler DL, Goldstein SA, Guilak F. Functional tissue engineering: the role of biomechanics. J Biomech Eng 2000;122(6):570–5.
24. Guilak F, Butler DL, Goldstein SA. Functional tissue engineering: the role of biomechanics in articular cartilage repair. Clin Orthop 2001;391(Suppl): S295–305.
25. Mauck RL, Soltz MA, Wang CC, *et al.* Functional tissue engineering of articular cartilage through dynamic loading of chondrocyte-seeded agarose gels. J Biomech Eng 2000;122(3):252–60.
26. Mauck RL, Seyhan SL, Ateshian GA, Hung CT. Influence of seeding density and dynamic deformational loading on the developing structure/function relationships of chondrocyte-seeded agarose hydrogels. Ann Biomed Eng 2002;30(8):1046–56.
27. van Susante JL, Buma P, van Beuningen HM, van den Berg WB, Veth RP. Responsiveness of bovine chondrocytes to growth factors in medium with different serum concentrations. J Orthop Res 2000;18(1): 68–77.
28. Pei M, Seidel J, Vunjak-Novakovic G, Freed LE. Growth factors for sequential cellular de- and re-differentiation in tissue engineering. Biochem Biophys Res Commun 2002;294(1):149–54.
29. Pei M, Solchaga LA, Seidel J, *et al.* Bioreactors mediate the effectiveness of tissue engineering scaffolds. Faseb J 2002;16(12):1691–4.
30. Blunk T, Sieminski AL, Gooch KJ, *et al.* Differential effects of growth factors on tissue-engineered cartilage. Tissue Eng 2002;8(1):73–84.
31. Jadin KD, Wong BL, Li KW, *et al.* Depth-associated variation in chondrocyte density in bovine articular cartilage during growth and maturation (Abstract). Trans Orthop Res Soc 2003;28:469.
32. Mauck RL, Nicoll SB, Seyhan SL, Ateshian GA, Hung CT. Synergistic action of growth factors and dynamic loading for articular cartilage tissue engineering. Tiss Eng. 2003;9(4):597–611.
33. Soltz MA, Ateshian GA. Experimental verification and theoretical prediction of cartilage interstitial fluid pressurization at an impermeable contact interface in confined compression. J Biomech 1998;31(10): 927–34.
34. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 1986;883(2):173–7.
35. Farndale RW, Sayers CA, Barrett AJ. A direct spectrophotometric microassay for sulfated glycosaminoglycans in cartilage cultures. Connect Tissue Res 1982; 9(4):247–8.
36. Stegemann H, Stalder K. Determination of hydroxyproline. Clin Chim Acta 1967;18(2):267–73.
37. Kelly TN, Wang CC-B, Mauck RL, Ateshain GA, Hung CT. Effects of seeding density and native pericellular matrix on the response of chondrocytes seeded in agarose hydrogels to dynamic deformational loading. Biorheology 2003 In Press.
38. Masuda K, Sah RL, Hejna MJ, Thonar EJ. A novel two-step method for the formation of tissue-engineered cartilage by mature bovine chondrocytes: the alginate-recovered-chondrocyte (ARC) method. J Orthop Res 2000;21(1):139–48.
39. Davis JM, ed. Basic Cell Culture: A practical approach. Oxford: Oxford University Press; 1996.
40. Bonassar LJ, Grodzinsky AJ, Frank EH, Davila SG, Bhaktav NR, Trippel SB. The effect of dynamic compression on the response of articular cartilage to insulin-like growth factor-I. J Orthop Res 2001;19(1): 11–7.
41. O'Hara BP, Urban JP, Maroudas A. Influence of cyclic loading on the nutrition of articular cartilage. Ann Rheum Dis 1990;49(7):536–9.
42. Mauck RL, Hung CT, Ateshian GA. Modeling neutrally charged solute diffusion in dynamically loaded porous permeable gels: applications for cartilage biosynthesis and tissue engineering. J Biomech Eng 2003;125(5):602–614.
43. Allen RG, Burstein D, Gray ML. Monitoring glycosaminoglycan replenishment in cartilage explants with gadolinium-enhanced magnetic resonance imaging. J Orthop Res 1999;17(3):430–6.
44. Banes AJ, Tsuzaki M, Hu P, *et al.* PDGF-BB, IGF-1 and mechanical load stimulate DNA synthesis in avian tendon fibroblasts in vitro. J Biomech 1995; 28(12):1505–13.
45. Gooch KJ, Blunk T, Courter DL, Sieminski AL, Vunjak-Novakovic G, Freed LE. Bone morphogenetic proteins-2, -12, and -13 modulate in vitro development of engineered cartilage. Tissue Eng 2002;8(4): 591–601.
46. Mouw JK, Case N, Guldberg R, Levenston ME. Scaffold dependent development of tissue engineered cartilage (Abstract). Ann Biomed Eng 2000;28(supp. 1): 121.
47. Sah RL, Grodzinsky AJ, Plaas AH, Sandy JD. Effects of tissue compression on the hyaluronate-binding properties of newly synthesized proteoglycans in cartilage explants. Biochem J 1990;267(3):803–8.
48. Hardingham TE. The role of link-protein in the structure of cartilage proteoglycan aggregates. Biochem J 1979;177(1):237–47.
49. Kim YJ, Grodzinsky AJ, Plaas AH. Compression of cartilage results in differential effects on biosynthetic pathways for aggrecan, link protein, and hyaluronan. Arch Biochem Biophys 1996;328(2):331–40.
50. Ragan PM, Chin VI, Hung HH, *et al.* Chondrocyte extracellular matrix synthesis and turnover are influenced by static compression in a new alginate disk culture system. Arch Biochem Biophys 2000;383(2): 256–64.
51. Wu JJ, Woods PE, Eyre DR. Identification of cross-linking sites in bovine cartilage type IX collagen reveals an antiparallel type II-type IX molecular relationship and type IX to type IX bonding. J Biol Chem 1992;287(32):23007–14.
52. Lawler J, Chen H. Cartilage Oligomeric Matrix Protein. Encyclopedia of Molecular Medicine: John Wiley & Sons, Inc.; 2002: p. 481–484.
53. Chen H, Lawler J. Cartilage oligomeric matrix protein is a calcium-binding protein, and a mutation in its type 3 repeats causes conformational changes. J Biol Chem 2001;275(34):26538–44.

54. Wong M, Siegrist M, Cao X. Cyclic compression of articular cartilage explants is associated with progressive consolidation and altered expression pattern of extracellular matrix proteins. Matrix Biol 1999; 18(4):391–9.
55. Giannoni P, Siegrist M, Hunziker EB, Wong M. The mechanosensitivity of cartilage oligomeric matrix protein (COMP). Biorheology 2003;40(1,2,3):101–9.
56. Quinn TM, Schmid P, Hunziker EB, Grodzinsky AJ. Proteoglycan deposition around chondrocytes in agarose culture: construction of a physical and biological interface for mechanotransduction in cartilage. Biorheology 2002;39(1-2):27–37.
57. Soltz MA, Ateshian GA. A Conewise Linear Elasticity mixture model for the analysis of tension-compression nonlinearity in articular cartilage. J Biomech Eng 2000;122(6):576–86.
58. Wang CC-B, Soltz MA, Mauck RL, Valhmu WB, Ateshian GA, Hung CT. Comparison of the equilibrium axial strain distribution in articular cartilage explants and cell-seeded alginate disks under unconfined compression (Abstract). Trans Orthop Res Soc 2000;25:131.
59. Hung CT, Lima EG, Mauck RL, *et al.* Anatomically shaped osteochondral constructs for articular cartilage repair. J Biomechanics 2003;0:00 in press.
60. Mauck RL, Nicoll SB, Stark R, Hung CT, Ateshian GA. Anatomically shaped molds for articular cartilage tissue engineering (Abstract). Trans Orthop Res Soc 2002;27:251.